MEDICAL SCIENCE MONITOR

ANIMAL STUDY

e-ISSN 1643-3750
© Med Sci Monit, 2017; 23: 5480-5487
DOI: 10.12659/MSM.907160

Received: 2017.09.16
Accepted: 2017.10.26
Published: 2017.11.18

Notch1 Signaling Activation Contributes to Adult Hippocampal Neurogenesis Following Traumatic Brain Injury

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Statistical Analysis C
Data Interpretation D
Manuscript Preparation E
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Source of support: This project was supported by grants from the Zhejiang Provincial Natural Science Foundation of China (LY13H090009) and the National Natural Science Foundation of China (80371370)

Background: Neural stem cells are reported to exist in the hippocampus of adult mammals and are important sources of neurons for repair. The Notch1 signaling pathway is considered as one of the important regulators of neural stem cells, but its role in adult brains is unclear. We aimed to describe the role of Notch1 signaling in the adult rat hippocampus after traumatic brain injury.

Material/Methods: The model rats were randomly divided into 4 groups as follows: sham, sham-TBI, sham-Ad-TBI, and NICD-Ad-TBI. We used adenovirus-mediated gene transfection to upregulate endogenous NICD *in vivo*. Firstly, a TBI rat model was constructed with lateral fluid percussion. Then, the hippocampus was collected to detect the expression of Notch1 markers and stem cell markers (DCX) by Western blot analysis, immunohistochemistry, and immunofluorescence. The prognosis after TBI treatment was evaluated by the Morris Water Maze test.

Results: First, we found the expression of NICD *in vivo* was significantly increased by adenovirus-mediated gene transfection as assessed by Notch1 immunofluorescence and Western blot analysis. Second, enhancing NICD stimulated the regeneration of neural stem cells in the DG of the adult rat brain following traumatic brain injury, as evaluated by DCX and NeuN double-staining. Furthermore, Notch1 signaling activation can promote behavioral improvement after traumatic brain injury, including spatial learning and memory capacity.

Conclusions: Our findings suggest that targeted regulation of Notch1 signaling may have a useful effect on stem cell transformation. Notch1 signaling may have a potential brain-protection effect, which may result from neurogenesis.

MeSH Keywords: Brain Injuries • Neurogenesis • Receptor, Notch1

Full-text PDF: https://www.medscimonit.com/abstract/index/idArt/907160

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Background

Neurogenesis occurs in the hippocampus regions of adult mammals in the subgranular zone (SGZ) of the hippocampus and the subventricular zone (SVZ) of the lateral ventricles [1,2]. Those regions are mainly due to the presence of neural stem cells (NSE), which are characterized by self-renewal and multilineage differentiation. Newly generated cells can differentiate into functional neurons and neural networks, which participate in the formation of cognitive function and emotion [3,4]. More recently, experiments revealed that TBI may stimulate stem cells with the unique ability to replace damaged neurons locally.

Notch signaling, composed of a series of molecular events, is an evolutionarily conserved signaling transduction pathway. At present, 4 Notch receptors have been found: (Notch 1–4) and 2 ligands: Jagged (Jagged 1, Jagged 2) and Delta-like ligands (Delta-like 1, Delta-like 3, and Delta-like 4) [5,6]. The Notch signaling pathway is usually activated when the extracellular domain of a Notch receptor expressed on one cell binds to Notch ligands expressed on its signaling cells in an interaction. A notch receptor intracellular domain (NICD) transports to the nucleus to regulate gene transcription, which affects the differentiation and development of regulated cells [7]. Researchers have revealed that the Notch signaling pathway is involved in regulation of stem cell activity, including stem cell-like features and differentiation [8,9]. However, the role of Notch signaling in promoting tissue regeneration and post-injury repair has been largely overlooked.

In our study, we researched the role of Notch1 signaling in neurogenesis by NICD activation following traumatic brain injury. Our results reveal the relationship between the activation of NSCs and Notch signaling pathways after TBI. These findings suggest that targeted regulation of Notch1 signaling may have a useful effect on stem cell transformation.

Material and Methods

Animals and grouping

All animal experiments were carried out according to the experimental animal health care and use guidelines of the Wenzhou Medical University Animal Experimental Center, and in accordance with relevant ethics standards. We obtained the adult male Sprague Dawley rats from the Slrc Laboratory Animal Center, Shanghai, China, approximately 6–8 months old and weighing 250±20 g. All the animals were maintained at 24°C for 12 h light/dark cycle and allow free access to food and water, and were acclimated for 1 week. We randomly assigned these rats into 4 groups as follow: rats without treatment (sham); rats treated by surgery with lateral fluid percussion (LFP) (n=20, sham-TBI group), rats injected with adenovirus and treated with surgery with LFP (n=20, sham-Ad-TBI group), and rats injected with the NICD overexpression adenovirus and treated with surgery with LFP (n=20, NICD-Ad-TBI group).

Construction the adenovirus of overexpressing NICD gene

We obtained the NICD cDNA fragment (5456 to 7819 nucleotides; NM_001105721(1747-2531aa)) from the cDNA library of Genechem (Shanghai, China). The primers according to the library were: 5'-GGA GGT AGT GGA ATG GAT CCC GCC ACC ATG TCC CGC AAG CGC AGG CGG CAG CAT GGC-3' and 5'-TCA TCC TTG TAG TCG CTA GCC TTA AAT GCC TCT GGA ATG TGG GTG-3'. The amplified fragment was cloned into a linearized adenovirus plasmid GV411 (Genechem) with T4 DNA ligase. The NICD overexpression adenovirus (NICD-Ad) was packaged in AAV-293 cells and purified with an Adeno-X™ Virus Purification Kit (BD Biosciences, San Jose, CA, USA). The endpoint dilution method was used to determine the viral titre. Adenovirus particles expressing a scrambled sequence (NC-Ad; purchased from Genechem) served as a negative control.

Stereotaxic injection of the adenovirus

For the injection of the adenovirus, adult rats were anesthetized with an intraperitoneal injection of 10% chloral hydrate (4 ml/g) and immobilized on a stereotaxic instrument (Stoelting, USA). Injection points (2.0 mm right to sagittal suture and 1.5 mm anterior to the rear of anterior fontanel) in the cranium was drilled with a dental drill, and a microsyringe needle was inserted 4.0 mm deep into the dorsal hippocampus (AP, 1.9 mm; L, 1.2 mm; DV, 1.8 mm [D], coordinates according to bregma) [10,11]. Ten microliters (containing 10^8 PFU Ad) of recombinant adenovirus was injected as previously described [12].

The construction of model rats

After Ad-NICD transfection and NC-Ad injection, all rats were anesthetized by intraperitoneal injection of 10% chloral hydrate (4 ml/g), then fixed on a stereoscopic device, and we prepared all surgical instruments. For the TBI experiments, the rat surgical incision was on the right side of the parietal bone and we made a 3-mm craniotomy using an electric drill (Harvard Apparatus). The lateral fluid percussion injury model has been previously described in detail [13]. The room temperatures were maintained at 37.0°C for the entire procedure.

Western blot

The hippocampi were dissected from rat brains on ice, and centrifuged at 15 000 rpm for 15 min at 37°C. The supernatant fluid was collected for Western blot analysis. Total protein was measured using the Protein Extraction Reagent Kit

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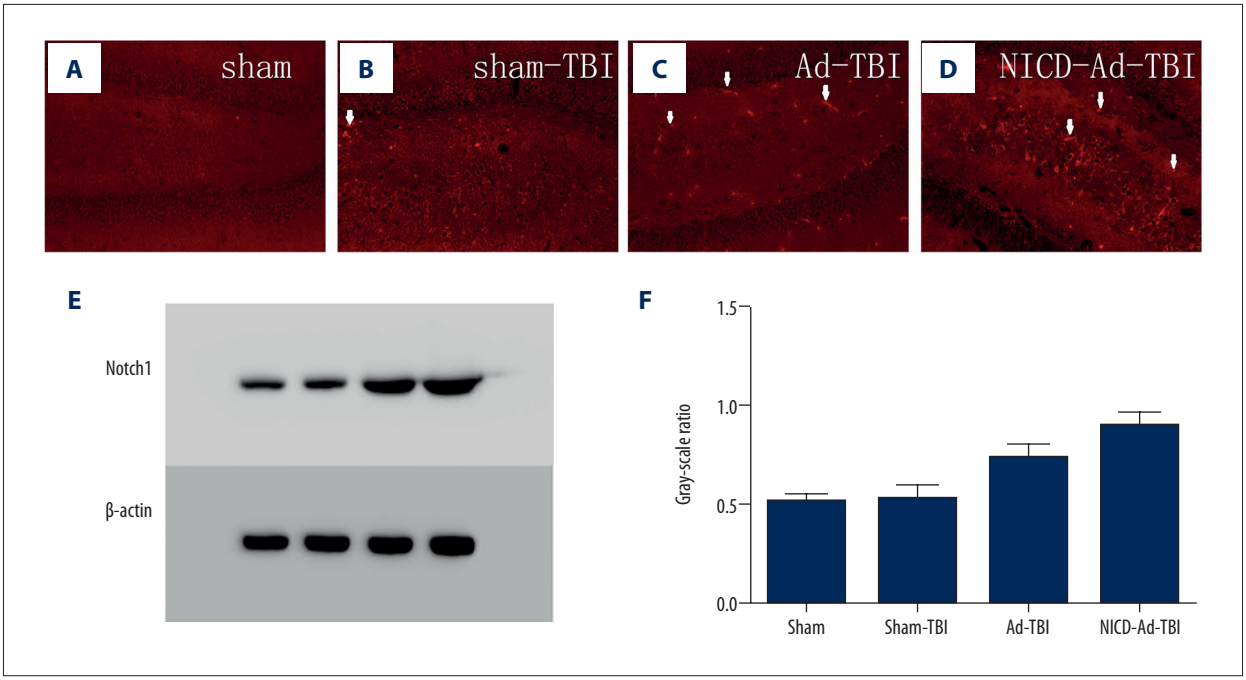

Figure 1. The expression of Notch1 in the rat brain. Adult rat coronal brain sections from 4 groups stained with anti-Notch1 antibody (red) (**A–D**). Notch1-expressing cells (white arrowheads) in adult hippocampal DG was significantly increased following transfection. (**E, F**) Western blot analysis of Notch1 expression in adult hippocampal DG from 4 groups. $P<0.05$.

(Takara Biotechnology, China). The proteins underwent electrophoretic separation on 12% SDS-PAGE gels, then were transferred into polyvinyl difluoride membranes. Membranes were then incubated overnight at 4°C using 1 of the following primary antibodies: an anti-Hes1 antibody (1: 800 dilution; Abcam, Cambridge, MA, USA), an anti-DCX antibody (1: 500 dilution; Abcam, Cambridge, MA, USA), and an anti-Notch (1: 1000 dilution; Abcam, Cambridge, MA, USA). Membranes were washed 3 times using TBST, for 5 mins each time. Then, membranes were incubated at room temperature for 60 min using a goat anti-rabbit secondary antibody. Secondary antibodies (diluted by TBST) were then incubated at 37°C for 1 h. After exposure, developing, and fixing, images were analyzed using an image analysis system. All results were referenced by beta-actin.

Immunohistochemistry

Rat brains were fixed, embedded in paraffin overnight at 4°C, then cut into 6-μm-thick sections. The sections were deparaffinized, rehydrated, and rinsed with PBS, followed by antigen retrieval in citrate buffer (pH 6.0) for 15 min at 95–100°C. Subsequently, non-specific antigens were blocked in 5% donkey serum for 60 min at 25°C. Then, sections were incubated with primary antibodies as follow: (1) goat polyclonal anti-Notch1 (Santa Cruz Biotechnology, Santa Cruz, CA; 1: 100); (2) goat polyclonal anti-Jagged1 (Santa Cruz Biotechnology; 1: 100); (3) rabbit polyclonal anti-Hes-1 (1: 800 dilution; Abcam, Cambridge, MA, USA); (4) goat polyclonal anti-DCX (1: 200 dilution; Abcam, Cambridge, MA, USA); After incubation, the sections were stained with the nuclear stain 4',6-diamidino-2-phenyl-indole (DAPI). A fluorescence microscope (Nikon, Japan) was used to observe, evaluate, and photograph these sections.

Double or triple immunostaining

The sections were fixed for 1 h and we used PBS to wash the sections twice, then the sections were incubated to denature DNA. Sections were incubated with blocking solution, then with primary antibodies at 4°C overnight, and with secondary antibodies in blocking solution at room temperature for 2 h. The primary antibodies were those listed above. The secondary antibodies were rhodamine-conjugated rat-absorbed donkey anti-mouse IgG (Jackson ImmunoResearch Laboratories Inc., West Grove, PA, USA; 1: 200) and fluorescein isothiocyanate (FITC)-conjugated pig anti-goat or goat anti-rabbit IgG (Jackson ImmunoResearch; 1: 200). The fluorescence images were acquired using an inverted Olympus fluorescence microscope and an image capture system.

Morris Water Maze (MWM) test

The Morris Water Maze (MWM) test is an experiment to investigate brain function, mainly used to test the learning and memory ability in spatial sense and direction perception [14,15]. It mainly forces rats learn to find a platform hidden in the water [16]. The escape platform is a 10-cm platform located in

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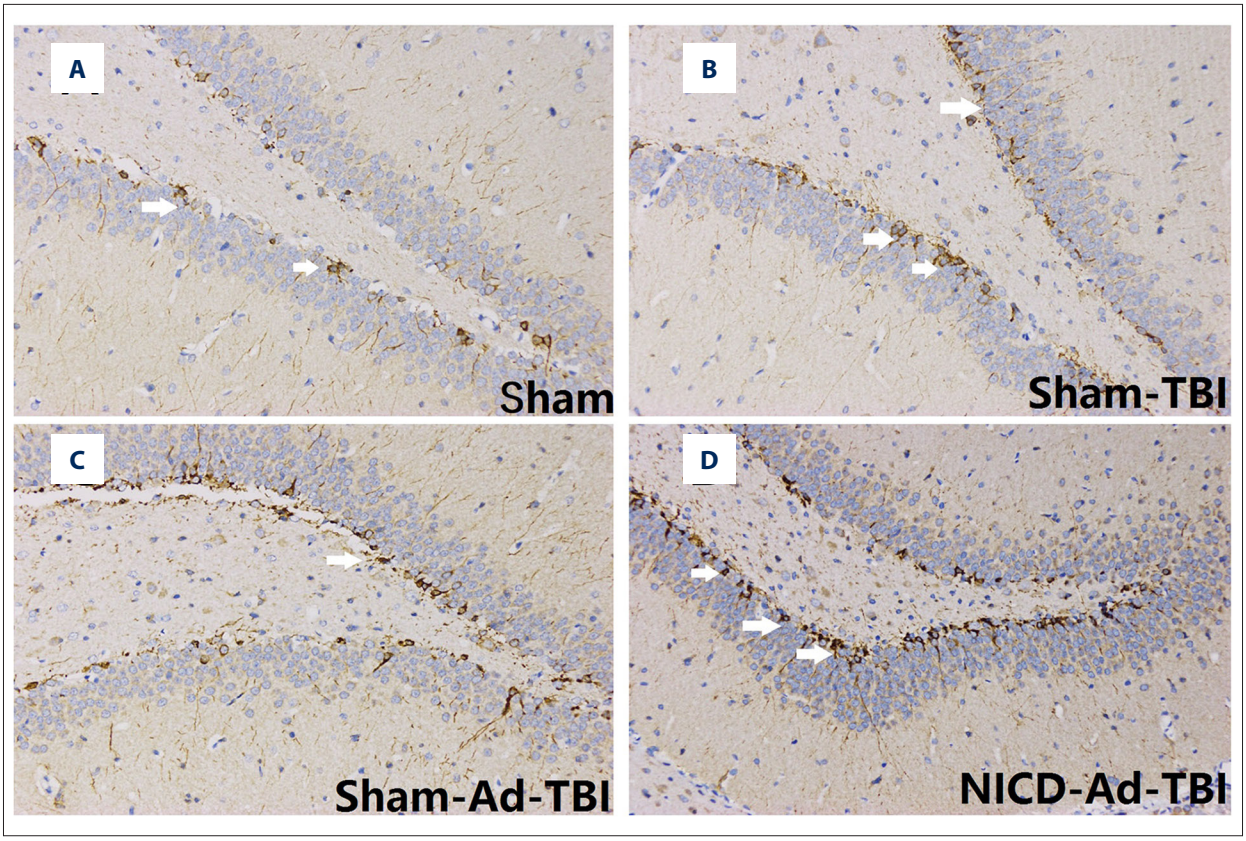

Figure 2. (**A–D**) The expression of neural stem cell markers in model rats. The hippocampus was dissected up to 7 days after the successful construction of model rats. Compared with the sham group, it was found that DCX+ cells increased obviously after traumatic brain injury. In addition, the results of this immunohistochemical staining clearly show that DCX+ cells proliferated significantly in the hippocampus after NIICD-adenoviral vector transfection. *P*<0.05.

the target quadrant, which is submerged 0.5 cm below the surface of the water. All tests were carried out in a pool filled with water 1.2 meters in diameter and filled with water made opaque with white non-toxic paint, connected to a computer via a digital camera to record the route, quadrant staying time, and quadrant searching time. The water was maintained at 25±2°C.

The rats were trained in the pool for 4 days before the injury, and we gave the second training from 3 days after injury until 7 days after injury. The rats stayed on the platform for 10 s before the training trial. Then, the rats were placed into the water along the pool wall and started to search for the platform. The test ends when the rat finds the platform or the search duration exceeds 60 s. The rats were placed to rest on the platform for 10 s after the training trial. Rats that failed to search for the platform could stay on platform for additional 15 s. The EL is the time taken by the animal to move from the starting quadrant to finding the hidden platform in the target quadrant [17]. The final experiments were performed on the 7 and 14 days after injury. The platform was removed from the pool and the rats were placed in the opposite quadrant of the escape platform they were located in before and allowed to swim to explore the target quadrant for 60 s. The mean time spent in the target quadrant searching for the missing platform and the percentage time staying in the target quadrant were recorded. The mean time spent in the target quadrant in searching for the missing platform served as the evaluation of memory function.

Statistical analysis

The data are represented as means ±SD. The differences between groups were analyzed by one-way ANOVA, and differences were considered statistically significant at P<0.05.

Results

Successful overexpression of NICD in hippocampal DG following NICD-Ad injection

We initially recognized whether NICD was activated successfully and could be injected into the NICD-Ad hippocampus

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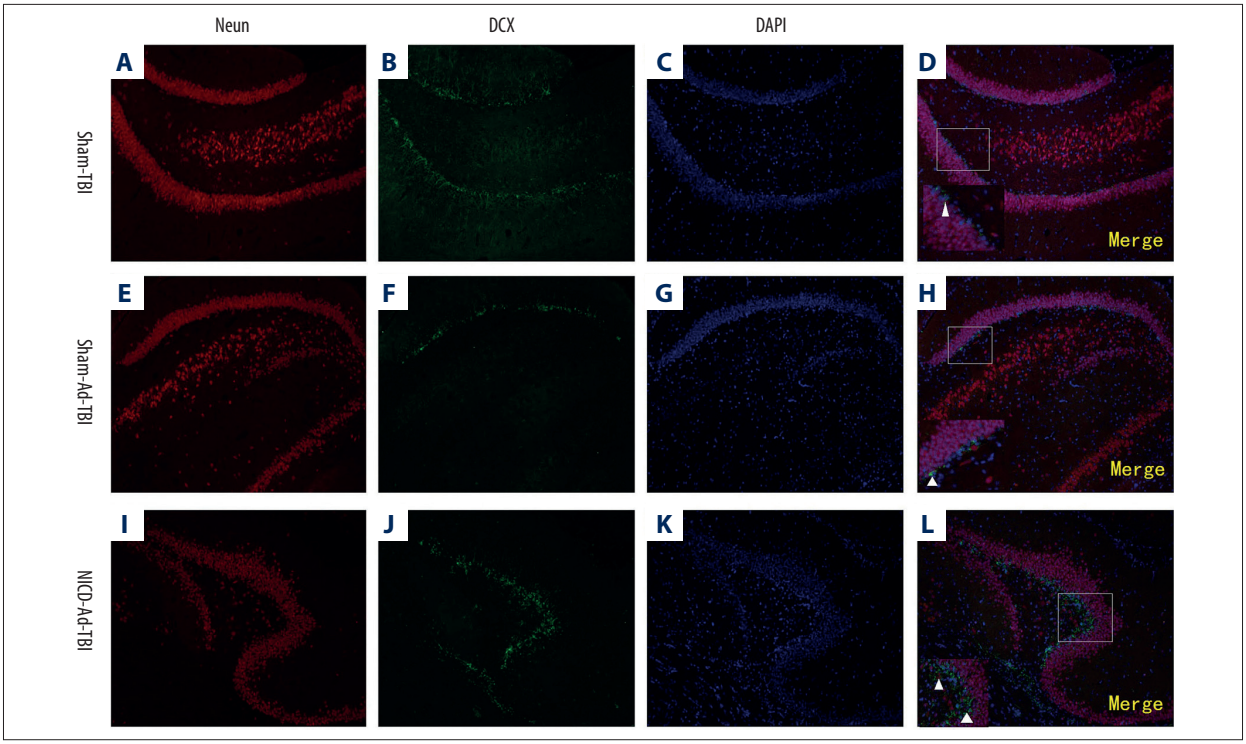

Figure 3. Regulation of Notch1 signaling significantly alters cell proliferation in normal adult rat brain. Immunostaining shows the increasing number of DCX+ cells on day 3 following TBI in NICD-Ad group, compared with sham-TBI (**A–D**) and NC-TBI (**E–H**) groups.

after effective transfection on the third day by immunofluorescence (Figure 1A–1D). The immunofluorescence staining confirmed that Notch1 was expressed in the hippocampus on the third day after traumatic brain injury. Compared with the other groups, the expression of Notch following NICD-Ad transfection was significantly increased (Figure 1D). We further confirmed the expression of Notch1 protein by Western blot quantitative analysis, and verified the level of Notch between different groups after NICD virus transfection (Figure 1E). The results revealed that Notch protein expression was significantly increased on the third day following NICD-Ad transfection after TBI. These results strongly suggest that NICD expression in SGZ in the adult rat hippocampus is regulated, and this also demonstrated the successful overexpression of Notch in the hippocampus DG following NICD-Ad injection.

Activation of Notch1 promotes hippocampal neurogenesis after TBI

To specifically verify the role of Notch1 signaling in the SGZ of adult rats, we activated Notch1 protein expression in neural precursors residing within the SGZ by NICD-Ad virus injection to the target region. To evaluate the effect of activation of Notch1 signaling on proliferation and differentiation of NSC in the SGZ, we used immunohistochemistry and fluorescence staining in the hippocampus region. The results of this immunohistochemical staining clearly show that DCX+ cells were significantly proliferated in the hippocampus after NIICD-adenoviral vector transfection (Figure 2). Immunostaining showed the increasing number of DCX+ cells on day 3 following TBI in NICD-Ad group, compared with sham-TBI (Figure 3A–3D) and NC-TBI (Figure 3E–3H) groups (Figure 3). These results strongly suggested that the activation of Notch1 gene affects the proliferation and differentiation of NPCs in the DG of the hippocampus after TBI.

Activation of the Notch1 signaling pathway promotes the improvement of spatial learning and memory in rats after TBI

To further study the effect of activation of Notch1 signaling in adult neurogenesis, we investigated animal behavioral changes following TBI. The NICD-Ad group showed cognitive improvement in rats with significant reduction in delayed detection platform on day 7 compared with the sham-TBI and Ad-NC groups ($P<0.05$; Figure 4). Then, we assessed the spatial memory capacity of rats by measuring the percentage of rats in the target quadrant during the probe trial by swimming test and the time spent searching for the platform. Compared with the sham-TBI and Ad-NC groups, the percentage of the mean time increased in the NICD-Ad group at 14 days after injury and the mean time of searching the platform was much

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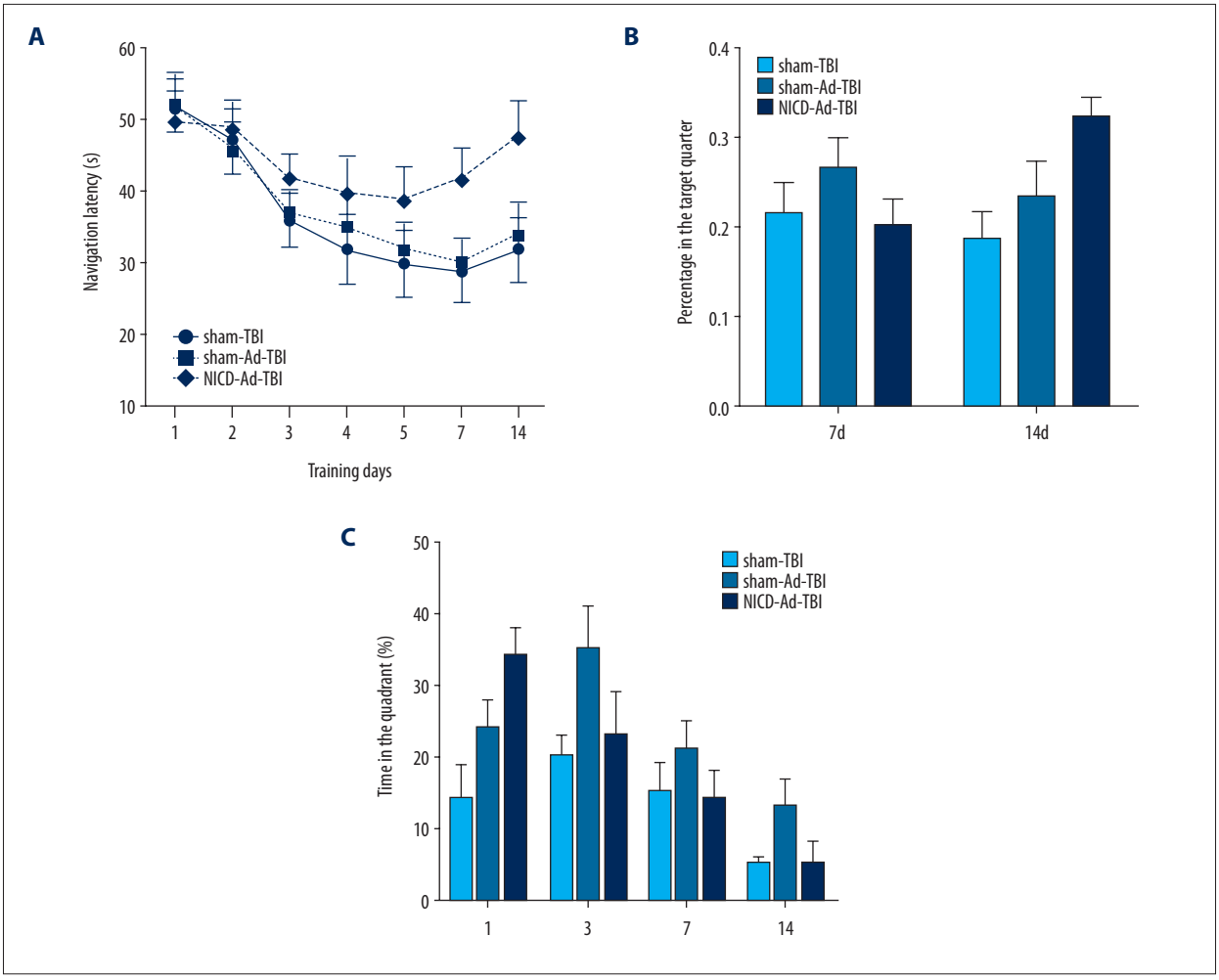

Figure 4. The alteration of spatial learning and memory in model rats after TBI. (**A**) The cognitive function of the experimental rats decreased significantly after injury. Compared to other groups, the NICD-Ad group recovered more quickly and the escape latency time significantly decreased (P<0.05). (**B**) The diagram represents the memory function through the time that the rats stayed in the target quarter during the probe trial. The results revealed the sham-Ad-TBI group in the target quarter was significantly higher than sham group and NICD-Ad group on day 7 after brain injury and the NICD-Ad group recovered on day 14 after brain injury. (**C**) The diagram also represents the memory function through the time spent searching for the platform in the target quarter. On day 1 after brain injury, the NICD-Ad group had the longest time spent searching in the target quarter. The rats treated with NICD-Ad had the shortest time needed to find the platform on day 3 after brain injury compared to the sham-Ad group rats (P<0.05).

less after day 3 after injury (P<0.05; Figure 4). Our results for activation of the Notch1 signaling pathway showed promoting effects on the improvement of spatial learning and memory in rats following TBI.

Discussion

In this study, the main results were that Notch1 signaling pathways regulate hippocampal neurogenesis and differentiation in the adult rat brain *in vivo* following traumatic brain injury. Notch1 signaling is activated by the use of Ad vector systems, and previous studies have shown that the use of adenovirus vectors directly to target cells can effectively manipulate the cells by direct injection in the brain [11]. Next, we studied the expression and the effects of manipulating Notch1 signaling on proliferation and differentiation of NSC in the hippocampal zone. We found that the Notch signaling pathways involved in neurogenesis under physiologic conditions also contribute to the neurogenesis-promoting effect of injury.

The Notch signaling hypothesis was proposed decades ago. Notch signaling is a large transmembrane receptor protein, which plays an important role in cell fate, cell development, differentiation, proliferation, apoptosis, and adhesion [18]. Hitoshi et al. suggest that Notch1 is an important regulator

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of neural stem cell maintenance and self-renewal during the development stage [19]. An *in vitro* study showed that Notch1 (NICD) and a Notch signaling downstream target (Hes1) are expressed in SVZ cells after brain stroke [20]. Some studies in zebrafish and mice have proved that the mutations of Notch can affect neuronal development and neural plasticity [21,22]. In addition, Notch signaling has been shown in the development and progression of many diseases. Promising data were reported by Sha et al., showing that acute seizures induced by kainic acid (KA) promote the activation of Notch signaling pathway and promote neuronal excitation [23]. It was also reported that NICD1 levels increased and enhanced potentiation of neuronal death in simulated ischemia [24]. Despite these recent advances, the role of the Notch1 signaling pathway in nerve regeneration after traumatic brain injury is unclear. We demonstrated that up-regulation Notch 1 signaling is associated with a high level of NICD expression. We injected NICD transfection virus genome directly into target brain areas to stimulate the activation of endogenous Notch signaling pathways. Our results reveal the successful overexpression of Jagged1 via an adenoviral vector carrying NIICD cDNA.

Most NSCs are mitotically inactive in the adult nervous system. Kageyama [25] found that Notch signaling also does not oscillate in most NSCs, except those that activate and enter the cell cycle. However, the balance between activity and quiescence is crucial to maintain the NSC pool for later neuron production. Notch signaling inhibits NSC differentiation by repressing proneural genes through the regulation of Hes gene expression [26]. Also, Jagged1 has been shown to regulate the Notch signaling pathway and promote neurogenesis and angiogenesis [26–28]. Thus, Notch1 signaling may play a crucial role in neurogenesis following traumatic brain injury. The Morris Water Maze is used as a behavioral model to assess learning and memory; the reduction in EL during training and the increase in mean time consumed in the target quadrant during the search show that learning and memory are improved after TBI [16]. In the present study, the results of this test suggest that activation of the Notch1 signaling pathway improved the learning and memory capacity after TBI. Our findings largely agree with and expand these previous studies.

The present study has some limitations that should be mentioned. We revealed *in vivo* evidence concerning endogenous Notch1 signaling in the proliferation of NPC. However, exogenous stimulus Notch signaling pathway should be assessed in future studies. Additionally, the relationship between Notch signaling and other signaling pathways should be considered after traumatic brain injury.

Conclusions

Those findings show that targeted regulation of Notch1 signaling may have a useful effect on stem cell transformation. Notch1 signaling may have a brain-protection effect, which may result from neurogenesis.

Conflicts of interest

None.

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